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Production of cutinolytic esterase by filamentous bacteria

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W.F. FETT, C. WIJEY, R.A. MOREAU AND S.F. OSMAN. 2000. Thirty-eight strains of filamentous bacteria, many of which are thermophilic or thermotolerant and commonly found in composts and mouldy fodders, were examined for their ability to produce cutinolytic esterase (cutinase) in culture media supplemented with cutin, suberin or cutin-containing agricultural by-products. Initially, the ability of culture supernatants to hydrolyse the artificial substrate *p*-nitrophenyl butyrate was determined by spectrophotometric assays. Only one bacterium, *Thermoactinomyces vulgaris* NRRL B-16117, exhibited cutinolytic esterase production. The enzyme was highly inducible, was repressed by the presence of glucose in the medium and hydrolysed both apple and tomato cutins. Inducers included apple cutin, apple pomace, tomato peel, potato suberin and commercial cork. Unlike similar fungal enzymes, the *T. vulgaris* cutinolytic esterase was not inducible by cutin hydrolysate. The cutinolytic esterase exhibited a half-life of over 60 min at 70 °C and a pH optimum of $\geq 11{\cdot}0$. This study indicates that thermophylic filamentous bacteria may be excellent commercial sources of heat-stable cutin-degrading enzymes that can be produced by fermentation of low cost feedstocks.

INTRODUCTION

Cutin is a unique insoluble biopolymer made up of omega hydroxy and epoxyhydroxy C_{16} and C_{18} fatty acids (Walton 1990). Cutin is present with waxes on the outer aerial surfaces of plants. A second unique plant polymer is suberin. Suberin forms a protective barrier in tissues, which undergo secondary growth (e.g. woody stems, roots and underground storage organs). In contrast to cutin, suberin is located internally between the primary cell wall and the plasmalemma in cork cells. Suberin is also formed as a protective barrier by wound periderm. The chemical makeup of suberin is more complex than that of cutin. It consists of aliphatic domains resembling cutin and aromatic domains resembling lignin (Walton 1990). The lipid component consists primarily of C_{12} to C_{34} fatty acids, fatty alcohols, omega hydroxy acids and dicarboxylic acids. The aromatic domain consists primarily of a covalently linked, hydroxycinnamic acid-derived polymeric matrix (Bernards *et al.* 1995). Many plant pathogenic fungi are reported capable of producing enzymes (cutinolytic esterases or cutinases) which can degrade cutin and several fungal cutinases have been purified and characterized (Koller 1991). In contrast, few bacteria appear capable of cutin degradation (Lin and Kolattududy 1980; Sebastian and Kolattukudy 1988; Fett *et al.* 1992a,b; Gray *et al.* 1995; Fett *et al.* 1999) and to our knowledge, there are no reports of degradation of suberin by enzymes of bacterial origin.

In this study we wished to determine if the ability to produce cutinolytic esterase is widespread among bacteria which exhibit filamentous growth, especially those that are thermophilic or thermotolerant and have vegetable composts and overheated fodders as their natural habitats. One such bacterium, *Thermomonospora fusca* ATCC 27730, produced a cutinolytic esterase (Fett *et al.* 1999). A second goal was to determine if low-value agricultural by-products such as tomato peel and apple pomace can be used as feedstocks for cutinase fermentations.

MATERIALS AND METHODS

Bacteria

The bacteria tested for cutinase production were *Saccharomonospora viridis* ATCC 15345, 15396, 15735 and 15736, and NRRL B-5791; *Saccharopolyspora erythraea* ATCC 11635 (type strain); *Saccharopolyspora hirsuta* subsp. *hirsuta* ATCC 27875 (type strain) and 27876; *Saccharopolyspora rectivirgula* ATCC 15347, 21450, 29034, 29035, 29681 and 33515 (type strain); *Streptomyces thermo-*

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diastaticus ATCC 27472 (type strain), *Streptomyces thermoflavus* ATCC 43907, *Streptomyces thermonitrificans* ATCC 23385 (type strain), 43902 and 43908; *Streptomyces thermophilus* ATCC 19282; *Streptomyces thermovialaceus* subsp. *apingens* ATCC 19994 (type strain); *Streptomyces thermovialaceus* subsp. *thermovialaceus* ATCC 19283 (type strain); *Streptomyces thermovulgaris* ATCC 19284 (type strain); *Thermoactinomyces candidus* ATCC 27868 (type strain) and 29680; *Thermoactinomyces vulgaris* ATCC 21364 and 43649 (type strain), NRRL B-2257, B-3130, B-3574, B-16177, B-16178, B-16179, B-16180, and B-16182; *Thermoactinomyces* sp. ATCC 14171, 14761 and 14762. All ATCC strains were obtained from the American Type Culture Collection (Rockville, MD, USA). All NRRL strains were obtained from Dr Dave Lebeda (USDA, ARS, National Center for Agriculture Utilization Research, Peoria, IL, USA).

For long-term storage bacteria were grown in Bacto tryptone-yeast extract liquid medium (TYE) (Difco, Detroit, MI, USA) (pH 8·0) for 2–3 d at their optimum temperature for growth (30–50 °C), an equal volume of 40% (v/v) glycerol was added and the preparations stored at −80 °C.

Preparation of cutins and suberin

Cutin was prepared from various fruits and vegetables purchased at a local supermarket as described previously (Gerard *et al.* 1993). Apples used were cultivar Golden Delicious. The same method was used to obtain suberin-enriched preparations from the periderm (skins) of potatoes purchased at a local supermarket.

Screening for esterase and cutinase activity

Bacteria were screened with TYE liquid medium supplemented with 0·4% (w/v) apple cutin adjusted to pH 8·0 before sterilization as described previously (Fett *et al.* 1999).

Esterase activity of culture supernatants of all 38 strains was determined by a spectrophotometric assay with *p*-nitrophenyl butyrate (PNB) as substrate (Sebastian *et al.* 1987) as previously described (Fett *et al.* 1992a). Cutinase activity of culture supernatant fluids of *T. vulgaris* NRRL B-16177 was determined with apple cutin as substrate (Fett *et al.* 1999).

Cutinolylic esterase induction/repression

The cutin monomer 16-OH palmitic acid, cutin hydrolysate and various other natural products were compared with apple cutin for their ability to induce production of cutinolytic esterase by *T. vulgaris* strain NRRL B-16177. Olive oil and 16-OH palmitic acid were purchased from Sigma (St Louis, MO, USA). Cutin hydrolysate (obtained from apple cutin), tomato peel, potato suberin and apple pomace were prepared as described by Fett *et al.* (1999). The ability of these various materials to induce cutinolytic esterase production and the ability of glucose to repress the production of cutinase by *T. vulgaris* NRRL B-16177 was determined as previously described (Fett *et al.* 1999).

Temperature stability and pH optimum

The temperature stability and pH optimum of the cutinolytic esterase produced by *T. vulgaris* NRRL B-16177 was determined is described previously (Fett *et al.* 1999).

RESULTS

Cutinolytic esterase production

The ability of culture fluids to hydrolyse the ester bond of the colourless substrate PNB is preliminary evidence of the presence of cutinase (Kolattukudy *et al.* 1981). A total of 38 strains of bacteria were tested for production of esterase in liquid media containing apple cutin using this method. This collection primarily contained filamentous bacteria, the majority of which are thermophilic. Of the strains tested with or without cutin in the media, only *T. vulgaris* NRRL B-16177 gave esterase values of > 500 nmol min^{-1} ml^{-1} over the 7 d incubation period. Esterase activity was highly inducible with strain NRRL B-16177 exhibiting a 680-fold increase in esterase production (20 nmol min^{-1} ml^{-1} compared with 13 580 nmol min^{-1} ml^{-1}) when cutin was present in the medium.

To confirm the cutin-degrading ability of the esterase-containing culture fluids obtained from *T. vulgaris* NRRL B-16177, filter-sterilized (0·2 μm filter) active culture fluids were incubated in the presence of cutin from apple or tomato and release of cutin monomers monitored by HPLC (data not shown). The culture fluids exhibited cutinase activity against both types of cutin as indicated by the release of fatty acid monomers typical of apple (9,10,18-trihydroxyoctadecanoic acid and 10,16-dihydroxyhexadecanoic acid) and tomato cutin (10,16-dihydroxyhexadecanoic acid) (Holloway 1982). Monomer identity was confirmed by GC/MS. Controls contained no significant amounts of cutin monomers. The presence of the nonionic detergent *n*-octylglucoside (35 mmol l^{-1}) had no effect on cutinase activity.

Comparison of various natural products as inducers

Various natural products were tested for their ability to induce esterase/cutinase production by *T. vulgaris* NRRL B-16177. Tomato peel and apple pomace induced the high-

Table 1 Ability of various natural products to induce esterase production by *Thermoactinomyces vulgaris* NRRL B-16177

Inducer	Concentration	Relative esterase activity (%)*	
		Experiment 1	Experiment 2
Tomato peel	0·4% (w/v)	306†	255
Apple pomace	0·4% (w/v)	121	129
Apple cutin	0·4% (w/v)	100	100
Potato suberin	0·4% (w/v)	71	81
Commercial cork	0·4% (w/v)	36	61
Cutin hydrolysate	8 μg ml^{-1}	< 1	< 1
	80 μg ml^{-1}	< 1	< 1
Olive oil	0·04% (v/v)	No growth	No growth
16-OH palmitate	0·029 mmol l^{-1}	No growth	No growth

*At 4 d of incubation. Esterase activity measured as μmol *p*-nitrophenol released min^{-1} ml^{-1} culture supernatant fluid.
†Values shown are means of values for each experiment.

est levels of cutinolytic esterase production (Table 1). Olive oil and 16-hydroxypalmitic acid completely inhibited growth of the bacterium.

Cutinolytic esterase activity in clarified culture fluids from cultures grown in media containing apple cutin, apple pomace, tomato peel and potato suberin was confirmed by HPLC with cutins from apple cv. Golden Delicious and tomato as substrates. The protein contents of the culture fluids ranged from 2·1 mg ml^{-1} (no inducer) to 4·8 mg ml^{-1} (commercial cork as inducer). Enzyme-specific activity was higher for all the culture fluids when apple cutin was used as the substrate. Of the various inducers tested, enzyme-specific activity was greatest for apple cutin and potato suberin.

Catabolite repression

Glucose (1% w/v) repressed cutinolytic esterase production over the 6-d incubation period. Esterase values in the media containing both glucose and apple cutin were up to 50-fold greater than in the control cultures (media alone) compared with a 1200-fold increase in the presence of cutin but without glucose. The pH of the glucose-containing cultures (no $CaCO_3$ added) dropped to 6·0 by day 6 compared with a final pH of 8·7–9·0 for the treatments containing apple cutin alone, apple cutin with glucose and $CaCO_3$ and media alone.

Temperature stability and pH optimum

Esterase activity of culture supernatants was not significantly reduced by heating at 50 or 60 °C for up to 1 h (Fig. 1). The half-life at 70 °C was 30 min. Significant reduction of esterase activity was seen after heating at 80 or 90 °C for as little as 15 min. Cutinase activity demonstrated good heat stability between 50 and 70 °C (Fig. 2).

With the buffers used, the *T. vulgaris* cutinase exhibited optimal activity at pH ≥ 11·0. Controls contained only minor amounts of cutin monomers.

DISCUSSION

Of the 38 strains of filamentous bacteria tested, only *Thermoactinomyces vulgaris* strain NRRL B-16177 exhibited

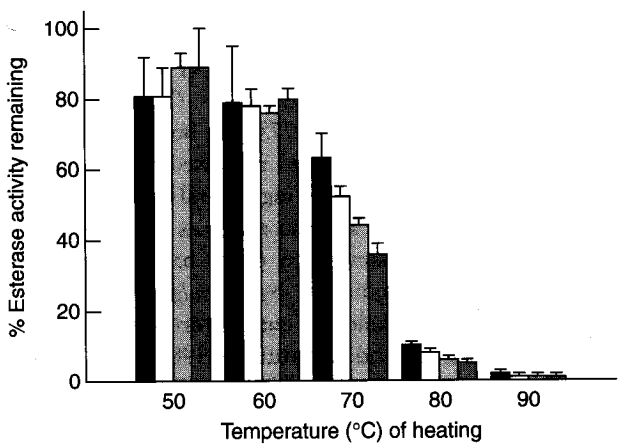

Fig. 1 Heat stability of esterase activity in induced culture supernatant fluids of *Thermoactinomyces vulgaris* NRRL B-16177 exposed for 15 min (■), 30 min (□), 45 min (▩) and 60 min (▩;). Esterase activity of the supernatant fluids was determined by spectrophotometric assay using *p*-nitrophenyl butyrate as substrate. Values shown are the average mean values from two experiments ± standard error

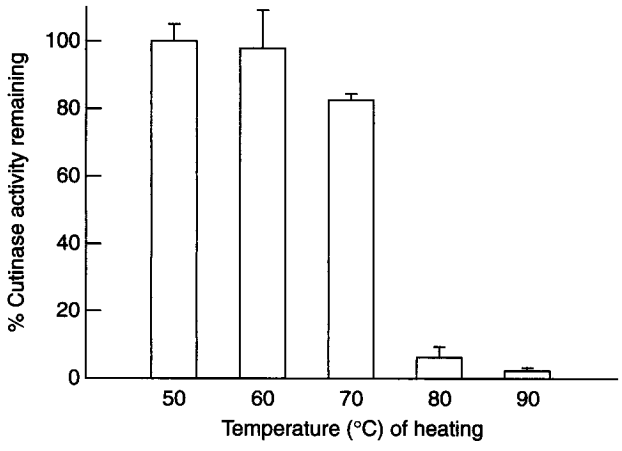

Fig. 2 Heat stability of cutinolytic esterase activity in induced culture supernatant fluids of *Thermoactinomyces vulgaris* NRRL B-16177. Culture fluids were heated for 60 min at the temperatures indicated. Apple cv. Golden Delicious cutin was used as the substrate. Values shown are the average mean values from two experiments ± standard error

cutinolytic esterase activity. *T. vulgaris* is an aerobic, saprophytic, thermophilic filamentous bacterium which grows well at 55 °C with a maximum growth temperature of 62 °C (Lacey 1989). This species is frequently isolated from decaying plant matter such as vegetable composts and mouldy fodders.

Based on the characteristics for the cutinolytic esterase produced by *T. vulgaris,* the enzyme appears to be very similar to a cutinolytic esterase produced by *Thermomonospora fusca* ATCC 27730 (Fett *et al.* 1999). The cutinolytic esterases produced by *T. vulgaris* NRRL B-16177 and *T. fusca* ATCC 27730 are both highly inducible by the addition of cutin and repressed by the presence of glucose. Other cutin-containing natural products (tomato peel and apple pomace) are also active as inducers. Unlike fungal cutinase, but similar to other bacterial cutinases, cutinolytic esterase production by *T. vulgaris* and *T. fusca* is not induced by inclusion of cutin hydrolysate (Sebastian *et al.* 1987; Sebastian and Kolattukudy 1988).

Under the experimental conditions utilized, the *T. vulgaris* and *T. fusca* cutinolytic esterases are similar in heat stability to the cutinase produced by the mesophilic bacterium *Pseudomonas* sp. ATCC 53553 and all three bacterial enzymes are considerably more heat stable than fungal cutinase (Sebastian *et al.* 1987). The *T. vulgaris* and *T. fusca* cutinolytic esterases have a very alkaline pH optimum (pH 11·0). The majority the fungal cutinases also have a basic pH optimum (pH 9·0) (Kolattukudy 1986), although fungal cutinases with pH optima below 7·0 have been reported (Koller and Parker 1989; Trail and Koller 1993). The bacterial cutinase produced by *Pseudomonas mendocina* has a broad pH optimum of 8·5–10·5 when tested against apple cutin (Sebastian and Kolattukudy 1988; Gray *et al.* 1995; Poulose and Boston 1996). The pH optimum of the cutinase produced by *Streptomyces scabies* has not been reported.

Induction of the cutinolytic esterase by potato suberin and commercial cork indicates that the *T. vulgaris* cutinase may have suberinase activity. When grown in the presence of suberin the fungus *Nectria haematococca* produces an esterase which can degrade suberin as well as apple cutin (Fernando *et al.* 1984). This esterase appeared to be very similar or identical to the previously characterized cutinases produced by this fungus.

Cutin-containing agricultural waste products such as apple pomace and tomato peel may represent low cost inducers for the commercial production of bacterial cutinase via fermentation.

ACKNOWLEDGEMENTS

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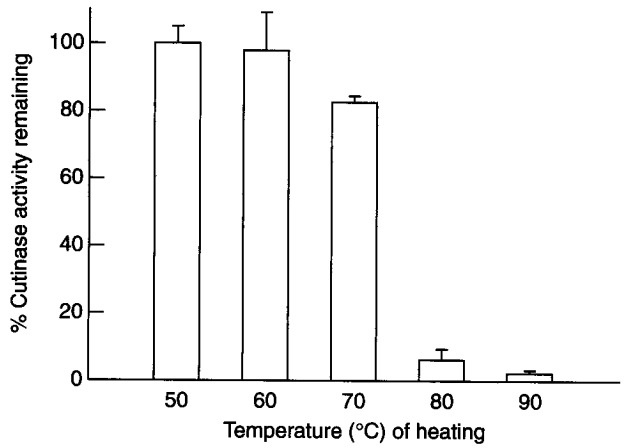

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